DIFFERENTIAL LPS BINDING TO DIABETIC AND CONTROL HUMAN FIBROBLASTS SUGGESTS MECHANISM FOR HIGH LEVELS OF INTERLEUKIN-8 EXPRESSION

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ABSTRACT

It is known that wound healing in the diabetic person is impaired and is characterized by excessive inflammation. However, the underlying mechanism(s) contributing to this heightened inflammatory response remain largely unknown. Previous studies from this laboratory, and others, have shown that fibroblasts can secrete pro-inflammatory mediators in response to exposure to lipopolysaccharide (LPS), a known inducer of inflammation. Further, fibroblasts obtained from type I diabetic individuals (GM01842) show significantly enhanced secretion of pro-inflammatory mediators compared to non-diabetic control cells (GM23973). This study had two primary aims, namely to investigate whether this increased secretion is due to elevated expression of CD14/TLR4 receptors, and whether LPS binding correlates with the expression of pro-inflammatory interleukin-8 (IL-8) versus extracellular matrix protein type I collagen (COL1A1).

Non-transformed human dermal fibroblasts from the Coriell Cell Repository were maintained under standard culture conditions. Cell binding conditions were optimized (temperature, time, and ligand concentration) for binding Alexa488-labelled LPS, and flow cytometry was used to quantify LPS binding. mRNA levels for COL1A1 and IL-8 were determined by RT-PCR using Bio-Rad SYP qPCR kits. Binding of LPS to GM01872 was shown to be dose dependent with 500 ng providing a significant increase in cell fluorescence. Of note, exposure of GM23973 cells to this LPS content suppressed fluorescence suggesting that the environment of the CD14 receptor may be altered. Given prior results and from the current study, IL-8 expression in GM01872 cells was significantly higher than GM23973 and whereas expression of COL1A1 was at non-treated levels.

These findings provide additional insights into the dysregulated inflammation observed in diabetic wound healing. CD14/TLR4 binding environments may be altered to facilitate higher levels of pro-inflammatory gene expression by cells that have experienced the hyperglycemic state of diabetes. These data offer new targets for therapeutic intervention.

INTRODUCTION

The repair of damaged tissue involves a complex interplay between numerous cell types and secreted mediators (Pena OA, Martin P. Nat Rev Mol Cell Biol. 2024;25(8): 599). In humans, damage in most tissues, including the skin, produces scar tissue rather than a replica of the original tissue, ie regeneration. However, in the context of various physiological states this fibrotic response is impaired, notably in people with diabetes mellitus (Armstrong DG, et al. JAMA 2023;330(1):62). The development of dermal ulcers in the diabetic is a known morbidity which can lead to serious tissue infections and potential amputation of lower extremity tissue. Our studies have been focused on what changes the diabetic, hyperglycemic state has produced in resident skin fibroblasts as the primary cells producing the major protein of scar – collagen. In particular, we have identified these cells as a significant source of pro-inflammatory mediators that are notably stimulated by the bacterial product lipopolysaccharide (LPS). The expression of the LPS binding receptor CD14/TLR4 on fibroblasts leads to a number of changes in these cells (Wang J, et al. J Cell Physiol 2011;226:1265). Our prior results have shown that while LPS drives a significant elevation in interleukin (IL)-1 6, and 8, and TNF-α the increased expression is even greater in fibroblasts isolated from type I diabetics. In some experiments, this expression exceeds that of non-diabetic fibroblasts by a factor of 1000.

The current study was undertaken to determine if the enhanced expression of IL-8 in diabetic-derived fibroblasts resulted from differential binding to the cells. We hypothesized that the diabetic fibroblasts possessed higher numbers of cell surface CD14/TLR4 binding sites. This could then lead to greater intracellular signaling to enhance gene expression. Additionally, given the higher expression of pro-inflammatory protein mediators by the diabetic cells, does this result in a reduced or blunted expression of the major extracellular repair protein collagen. We hypothesized that expression of type 1 collagen would not show a major increase in expression in response to LPS treatment.

RESULTS

Serum Concentration	Incubation Temperature (°C)	Fluorescence FL1-A
10%	4	54,086
	22	73,427
	37	-266,404
15%	4	17,262
	22	-389,262
	37	-258,069

Table 1. Optimization of LPS binding.
Binding of LPS to cell surface receptor CD14/TLR4 was optimized for serum concentration, temperature, labelled LPS, and time. This table provides a representative experiment concerning serum concentration and assay binding temperature.

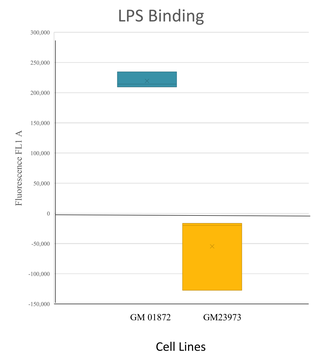


Figure 1. Fluorescence in FL1A channel for fibroblasts under optimized conditions.

Once binding conditions were optimized, direct comparison of cell surface binding between diabetic and non-diabetic cells was performed. As shown in Figure 1, there was a profound difference in fluorescence between the two fibroblast lines. This difference appeared to reflect a reduction below baseline in the control cells that might reflect differences in the binding environment.

Prior studies in our laboratory have shown for a number of cell lines that diabetic fibroblasts showed robust increases in pro-inflammatory mediators, including IL-8 (Figure 3). We asked the question, did this increase in inflammatory mediator expression also include the major ECM protein of fibroblasts, type I collagen expression. As shown in Figure 2, this difference does not hold.

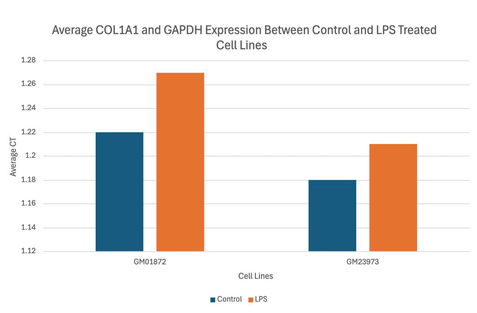


Figure 2. Expression of type I collagen mRNA by diabetic and control fibroblasts treated with LPS (1 µg/mL) for 24 hrs.

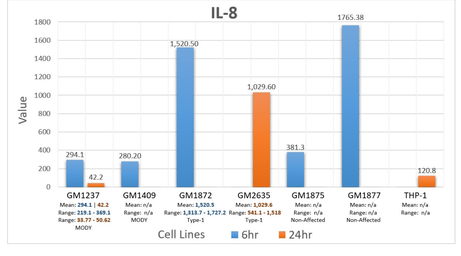


Figure 3. Expression of IL-8 by multiple cell lines treated with LPS (1 µg/mL) for 6 or 24 hrs. (THP-1 – inflammatory cell line)

METHODS

Cell culturing
- Non-transformed human dermal fibroblasts were obtained from the NIGMS cell bank at the Coriell Institute.
- Cells were grown to confluence in DMEM medium containing 10% FBS and penicillin/streptomycin.
- Cells were used between P7 & PP11 before signs of senescence of the cultures.

LPS Binding Studies
- Cells were incubated with Alexa Fluor 488-labeled LPS (Invitrogen) to assess binding, with varying LPS concentrations (25, 5 & 0.1 µg).
- Optimal conditions for LPS binding were determined by adjusting temperature, incubation time (15-30 minutes), fetal calf serum concentration, and ligand concentration to achieve the highest binding efficiency.
- Negative controls were used, including untreated cells and cells incubated with unlabeled LPS, to confirm specific binding.

Flow Cytometry Analysis
- After incubation with Alexa Fluor 488-labeled LPS, fibroblasts were harvested, washed, and resuspended in Dulbecco's PBS.
- Samples were analyzed using a flow cytometer (BD Accuri™ C6 Plus) to measure fluorescence intensity.
- For selected runs, cells were treated with Trueblack Autofluor Quencher (Biotium) to reduce non-specific background fluorescence.
- Negative controls set baseline fluorescence levels
- Alexa Fluor 488 fluorescence was measured at an emission wavelength of 520 nm following excitation at 488 nm.
- Appropriate gating was applied to exclude debris and dead cells using forward and side scatter parameters.

Expression of COL1A1
- Total RNA was extracted from cells cultured for 24 hrs. with 1.0 µg/mL LPS using a Monarch® Nucleic Acid Purification Kit (New England Biolabs).
- Quantitation of COL1A1 mRNA was by qRT-PCR using an iTaq Universal SYBR Green One-Step Kit (Bio-Rad Laboratories) with primers synthesized by IDT and GAPDH.
- Values are expressed as the ΔC_T value using GAPDH as the housekeeping gene.

CONCLUSIONS

These results provide further support for our earlier studies showing that human dermal fibroblasts from people with type diabetes respond differently to LPS. These data suggest that the hyperglycemic state may alter the CD14/TLR4 binding environment to engage a more robust signal resulting in enhanced pro-inflammatory signaling with out altering ECM expression. One could speculate that this response could lead to the impaired healing response that characterizes diabetes.